

Transcriptome sequencing to produce SNP-based genetic maps of onion

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Abstract We used the Roche-454 platform to sequence from normalized cDNA libraries from each of two inbred lines of onion (OH1 and 5225). From approximately 1.6 million reads from each inbred, 27,065 and 33,254 cDNA contigs were assembled from OH1 and 5225, respectively. In total, 3,364 well supported single nucleotide polymorphisms (SNPs) on 1,716 cDNA contigs were identified between these two inbreds. One SNP on each of 1,256 contigs was randomly selected for genotyping. OH1 and 5225 were crossed and 182 gynogenic haploids extracted from hybrid plants were used for SNP mapping. A total of 597 SNPs segregated in the OH1 × 5225 haploid family and a genetic map of ten linkage groups (LOD ≥ 8) was constructed. Three hundred and thirty-nine of the newly identified SNPs were also mapped using a

previously developed segregating family from BYG15-23 × AC43, and 223 common SNPs were used to join the two maps. Because these new SNPs are in expressed regions of the genome and commonly occur among onion germplasms, they will be useful for genetic mapping, gene tagging, marker-aided selection, quality control of seed lots, and fingerprinting of cultivars.

Introduction

In spite of its economic significance as one of the major vegetable crops worldwide, genetic maps of onion (*Allium cepa* L.) remain relatively rudimentary. This is due in part to the enormous onion genome, which at 16.3 gigabases per 1C nucleus (Arumuganathan and Earle 1991) makes identification of some molecular markers technically difficult, and the biennial generation time and severe inbreeding depression which slow the development of segregating families. Nevertheless, numerous classes of molecular markers have been developed and mapped in onion, including restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphisms (AFLPs), simple sequence repeats (SSRs), and single nucleotide polymorphisms (SNPs) (King et al. 1998; Van Heusden et al. 2000; Martin et al. 2005; Baldwin et al. 2012a, b). Molecular markers, such as SSRs and SNPs, are especially useful for onion because they are codominant and efficiently revealed by the polymerase chain reaction. Onion SSRs and SNPs have been used for cultivar identification (Jakše et al. 2005; Mahajan et al. 2009), genetic diversity estimates (Baldwin et al. 2012a), and tagging of chromosome regions affecting economically important traits such as fructan accumulation (Havey et al. 2004; McCallum et al. 2006; Raines et al. 2009), male-sterility

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restoration (Gökçe et al. 2002), and flavor (Galmarini et al. 2001; McCallum et al. 2007).

Marker-aided selection (MAS) has great potential in onion improvement because of the high cost of harvesting and vernalizing bulbs prior to flowering and completing crosses with insects. High throughput platforms that allow for the genotyping of large numbers of markers across individuals would allow breeders to select plants at an early stage of development and advance to seed production only a fraction of the numbers of plants as compared to classical phenotypic selection. SNPs are the marker of choice for MAS in onion because of codominance, common occurrence among elite germplasms (Martin et al. 2005; Baldwin et al. 2012b), and the availability of commercially available high-throughput genotyping platforms. In this research, we completed transcriptome sequencing of two inbred onion populations, identified SNPs in expressed regions of the onion genome, and extracted gynogenic haploid plants for genetic mapping of these SNPs. Our research has produced a large number of expressed sequences and the most detailed genetic maps based on codominant SNP markers developed to date for onion.

Materials and methods

RNA isolation and cDNA synthesis and normalization

Two long-day onion populations were selected for cDNA synthesis and SNP identification. 5225 is a red onion derived from a cross between North American Spanish and long-storage germplasms, was putatively a doubled haploid derived from the female gametophyte, and was the gift of Seminis Seed Company (Woodland, CA, USA). OH1 is a yellow inbred selected from long-day storage populations and shows relatively high production of gynogenic haploids (Havey and Bohanec 2007). At approximately 6 weeks after planting vernalized bulbs, tissue from leaves, unopened umbels, bulbs, and roots were separately harvested and frozen in liquid nitrogen. RNA extractions, cDNA syntheses, and normalizations were completed by BioS&T (Montreal, Canada). Total RNA was isolated separately from each tissue using the Trizol method (Invitrogen, Carlsbad, CA, USA). RNA concentrations were determined and equal amounts of RNA from each tissue were combined to create an RNA pool for each onion population. These two RNA pools were used for cDNA synthesis using the SMART (Switching Mechanism At 5' end of RNA Transcript) method (Clontech, Mountain View, CA, USA). Doubled stranded cDNAs were produced by extensions using 5' cDNA double-stranded adaptor 5'-CAGTGGTATCAACGCAGAGTGGCCATTA CGGC CTAGTT ACGGG-(cDNA)-3' and 3'-GTCACCATAGT

TGCGTCTCACCGGTAAT GCCGGATCAAT GCCC-(cDNA)-5'. The 3' cDNA double-stranded adaptor was 5'-cDNA-AAAAAAAAAAAAAAAAAGGCCGCCTCGG CC ACTCTGCGTTGATACCACTG-3' and 3'-cDNA-TTTT TGTGTGTGTTTCCGGCGGA GCCGGTGAGACGCAA CTATGGT GAC-5'. The amount and quality of cDNAs were established using agarose-gel electrophoresis and cDNAs over 0.5 kb in size purified from the gel. Normalization of cDNAs was completed by BioS&T using proprietary techniques.

Sequencing of cDNAs and identification of SNPs

The two cDNA libraries were individually barcoded and 2.5 plates (corresponding to 1.25 plate per population) were sequenced at the J. Craig Venter Institute (JCVI) using the Roche 454 FLX platform and protocols as recommended by the manufacturer (Roche, Branford, CN, USA). The 454 reads from 5225 and OH1 were assembled together using the Newbler Assembler (Roche). Well supported SNPs were identified between these two inbreds by keeping only single-base polymorphisms (ignoring indels and multi-base polymorphisms) and completing the filtering steps listed in Table 1. Sequences flanking these SNPs (approximately 60 basepairs on each side of SNP) were compared to the most similar rice genomic sequence to eliminate SNPs near introns; we previously demonstrated that 83 % of introns are shared between onion and rice (Martin et al. 2005). Sequences flanking SNPs were then analyzed by proprietary software programs for the Golden Gate (Illumina, La Jolla, CA, USA) and KASPar (LGC Genomics, Beverly, MA, USA) platforms to identify those conducive for genotyping using the respective platform. The assembled contigs from 5225 and OH1 were annotated by the JCVI EUK-auto-naming pipeline using databases of plant proteins from Swissprot and TrEmbl, NCBI NR, and UniRef100.

Extraction of haploids and confirmation of ploidy level

Single plants of OH1 and 5225 were crossed, seed was harvested from the OH1 plant, bulbs were produced in Wisconsin USA, and hybrids identified by red-bulb color. Hybrid bulbs were vernalized for 8 weeks at 5 °C and then shipped to Slovenia for gynogenic haploid extraction as previously described (Jakše et al. 2010). Haploid plants were allowed to form bulbs in a greenhouse in Slovenia. These bulbs were sent to the USA and planted in a greenhouse.

Young expanding leaf tissue was harvested, kept on ice, and immediately prepared for flow-cytometric analyses. Suspensions of nuclei were prepared using CyStain PI absolute P kit (Partec, Swedesboro, NJ, USA). Approximately 1 × 5 cm piece of each leaf was harvested and chopped using a sharp razor blade for 60–90 s in 2 ml

Table 1 Filtering steps used to identify well supported single nucleotide polymorphisms (SNPs) on cDNA contigs between OH1 and 5225

Step	Description	Number of SNPs	Number of SNP-carrying contigs
0	Output from 454 Newbler mapping	65,675	13,861
1	Read support: minimum three reads and 75 % of reads supporting individual SNPs in each inbred	12,527	3,955
2	Avoid contig ends: SNP required to be >100 nucleotides away from ends of contig	11,496	3,742
3	Selection against residual heterozygosity: if an inbred appeared to be heterozygous, the SNP was retained only when the most common nucleotide was at least three times more prevalent than the less common nucleotide	8,329	3,264
4	Rice exon mapping: SNP must be >50 nucleotides away from exon boundaries	3,364	1,716
5	SNPs amenable for KASPar genotyping	2,285	1,256

extraction buffer in a Petri dish. After incubation at 4 °C for 5 min, the buffer was filtered through a 30 µm Cell-Trics filter (Partec), and centrifuged for 5 min at 200 RCF. Pellets from individual plants were resuspended in 500 µl staining buffer containing propidium iodide and RNase as recommended by the manufacturer. The stained suspension was incubated at 37 °C for 1 h before analysis using a FACSCalibur flow cytometer with 488 nm Argon laser excitation at the Carbone Cancer Center, University of Wisconsin-Madison. At least 1,200 nuclei were counted per sample. Ploidy level was determined by comparing the histogram of each individual with diploid onions OH1 and 5225 using CellQuest Pro software (BD BioSciences, San Jose, CA, USA).

Marker genotyping

One SNP between 5225 and OH1 was identified on each of 1,256 cDNA contigs and was genotyped using the KASPar platform. DNAs used for SNP genotyping were isolated by CsCl purification (Bark and Havey 1995) from pooled leaf tissues from at least 25 plants from a diverse set of onion populations (Table 2) and from 182 haploid progenies extracted from hybrids between OH1 and 5225. We also genotyped SNPs using DNAs from 57 F₂-derived F₃ progenies from Brigham Yellow Globe 15–23 × Alisa Craig 43 (BYG15-23 × AC43); this family has been previously used for genetic mapping of RFLPs, SNPs, and SSRs (King et al. 1998; Martin et al. 2005). For haploid progenies selected from different F₁ plants, homogeneity of errors (Gomez and Gomez 1984, pages 464–467) was established before pooling segregations across families. Goodness-of-fit to the expected segregation ratios and genetic mapping using the regression algorithm and Kosambi function were completed with the JoinMap[®] software version 3 (Van Ooijen and Voorrips 2001) for each segregating family. JoinMap was also used to reveal synteny between the two genetic maps from OH1 × 5225 and BYG15-23 × AC43. Linkage groups were assigned to chromosomes based on previous assignments using the BYG15-23 × AC43 family (Martin

et al. 2005). Map images were drawn using the MapChart software (Voorrips 2002).

Results

Transcriptome sequencing and annotation

Sequencing of 2.5 454-plates (1.25 plates each for 5225 and OH1) yielded over 1 billion bases of expressed onion sequence (Table 3). The numbers of reads and total numbers of bases were similar for the two cDNA libraries. Sequences are available from Genbank's Sequence Read Archive accessions SRX188612 and SRX188621 for 5225 and OH1, respectively. Approximately 1.6 million reads from each library assembled into 27,065 and 33,254 contigs for OH1 and 5225, respectively, with average contig length of approximately 1.2 kb. Approximately 12 and 20 % of contigs were unique to OH1 or 5225, respectively. Individual Transcriptome Shotgun Assemblies (TSAs) were deposited at DDBJ/EMBL/GenBank under accessions GAAN000000000 and GAAO000000000, with the first versions described in this research as GAAN010000000 and GAAO010000000 for 5225 and OH1, respectively. About 7.5 % of reads from the combined assembly of OH1 and 5225 remained as singletons. Functional annotations of the cDNA contigs from the combined assembly are listed in Supplemental Table 1. The annotations of 14,357 of the 48,459 (30 %) cDNA contigs remained unknown (i.e., no hit to databases).

SNP identification

Initial assemblies revealed 65,675 SNPs between OH1 and 5225 on 13,861 cDNA contigs. We used the criteria listed in Table 1 to select well supported SNPs between OH1 and 5225, yielding 3,364 SNPs on 1,716 cDNA contigs (Supplemental Table 2) and an average of one SNP per 1.7 kb of expressed sequence. For the Illumina genotyping platform, 1,830 SNPs were identified with designability value

Table 2 Onion populations evaluated for single nucleotide polymorphisms

Population ^a	Characteristics ^b	Origin or citation ^c
BYG15-23	LD inbred with yellow bulbs	King et al. (1998)
5225	LD inbred with red bulbs	Seminis Seed Company
OH1	LD inbred with yellow bulbs	Havey and Bohanec (2007)
AC43	LD inbred with yellow bulbs	King et al. (1998)
2107	LD doubled haploid with yellow bulbs	Alan et al. (2007)
H6	SD doubled haploid with red bulbs	Texas A&M University
PI 239633	Population from Iran with a mixture of red, white, and yellow bulbs	USDA PGC
PI 248753	Population from India with red bulbs	USDA PGC
PI 248754	Population from India with red bulbs	USDA PGC
PI 255462	Population from Russia with yellow bulbs	USDA PGC
PI 258956	Population from Chile with yellow bulbs	USDA PGC
PI 264320	Population from Spain with yellow bulbs	USDA PGC
PI 274780	Population from India with red bulbs	USDA PGC
PI 288272	Population from India with red bulbs	USDA PGC
PI 289689	Population from Australia with yellow bulbs	USDA PGC
PI 321385	Population from Romania with yellow bulbs	USDA PGC
PI 546100	Sweet Spanish with yellow bulbs from Utah, USA	USDA PGC
PI 546115	Sweet Spanish with white bulbs from California, USA	USDA PGC
PI 546188	Sweet Spanish with yellow bulbs from California, USA	USDA PGC
PI 546192	Sweet Spanish with yellow bulbs from Michigan, USA	USDA PGC
PI 546303	Inbred 9885 with yellow bulbs and glossy foliage released by the USDA in 1985	USDA PGC
PI 546305	Inbred 9897 with yellow bulbs and glossy foliage released by the USDA in 1985	USDA PGC

^a Plant introduction (PI)^b LD and SD indicates long- or short day-length response for bulb formation, respectively. Sweet Spanish refers to onions commonly grown in the western USA with relatively low pungency and large size^c PGC plant germplasm collection**Table 3** Sequencing results from 1.25 454-plates from each of two onion inbreds (OH1 and 5225)

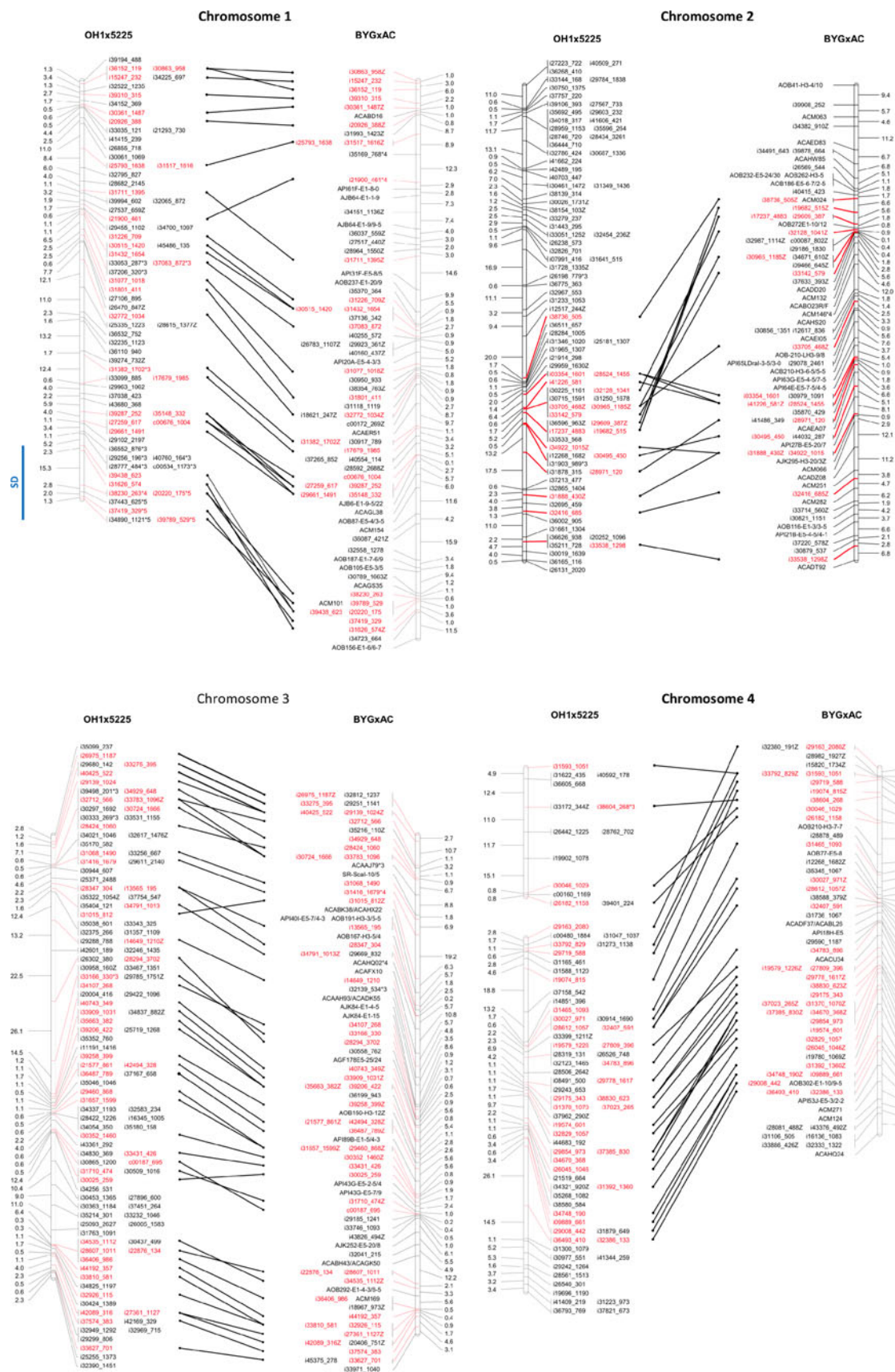
Characteristic	Assemblies		
	OH1	5225	Combined
Total number of reads	1,653,359	1,623,738	3,277,097
Total number of bases	564,222,318	520,384,051	1,084,606,369
Contigs assembled	27,065	33,254	48,459
Average length of contigs in bases	1,185	1,296	1,316
Total size of contigs in bases	32,061,028	43,107,082	63,747,620
Remaining singletons	134,417	168,959	227,140
Average singleton length in bases	331	308	311
Total bases of singletons	44,436,985	51,937,142	70,660,354

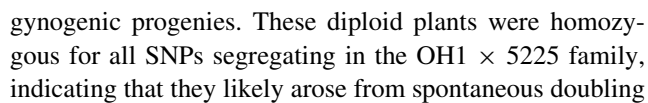
of 1 (Supplemental Table 3). For the KASPar genotyping platform, 2,285 SNPs were identified on 1,256 cDNA contigs that were conducive to genotyping using this platform (Supplemental Table 4). One SNP from each of the 1,256 cDNA contigs was randomly selected for genotyping using the KASPar platform (Supplemental Table 5). Of these 1,256 primer sets, 930 produced amplicons and revealed the expected SNPs across the diverse onion populations (Table 2, Supplemental Tables 6 and 7). From these successful amplifications, OH1 and 5225 were heterozygous for 9.8 and 19.9 % of the SNPs; in contrast amplicons from doubled haploids 2107 and H6 which appeared heterozygous for only 1.4 and 1.3 % of the SNPs (Supplemental Table 7).

Extraction of haploids and genetic mapping

Over 400 gynogenic haploids were obtained from 25 hybrid plants from the cross OH1 by 5225. Flow cytometry identified 11 diploid plants out of a random sample 96

Fig. 1 Genetic maps of single nucleotide polymorphisms (SNPs) segregating in the OH1 × 5225 haploid family (linkage groups on *left*) and restriction fragment length polymorphisms, simple sequence repeats, and SNPs in the BYG15-23 × AC43 family (linkage groups on *right*). Genetic distances are in centiMorgans (cM). SNPs segregating in both families are in *red* and *lines* correspond to their relative positions in the two maps. Chromosome assignments are based on markers previously assigned to chromosomes using the BYG15-23 × AC43 map (Martin et al. 2005). *Blue bars* indicate markers showing significant segregation distortion ($P < 0.001$). Marker names followed by an *asterisk and number* (*3, *4, and *5) indicate significant distortion from expected ratios at $P < 10^{-3}$, $P < 10^{-4}$, and $P < 10^{-5}$, respectively (color figure online)





of a cell from the female gametophyte. One hundred and eighty-two haploids (48, 44, 32, 30, and 28 haploids extracted from five different hybrid plants) were selected

for genetic mapping. Of the 930 primer sets that produced amplicons, 178 were excluded because they did not segregate (158) or produced heterozygous genotypes (20), likely due to amplifications from paralogs, among the haploid progenies. Of the 752 remaining amplicons, 522 (69 %) SNPs fit the expected 1:1 ratio at $P > 0.001$. For the 230 (31 %) SNPs not fitting the expected 1:1 ratio at $P > 0.001$, 155 were eliminated because of highly skewed segregation ratios at $P < 0.00001$. Errors were homogeneous ($P > 0.01$) for the segregating SNP markers across haploids extracted from independent hybrid plants; therefore segregations were pooled across families.

Genetic mapping of 597 SNPs using the haploid progeny DNAs from OH1 \times 5225 yielded 10 linkage groups at LOD ≥ 8.0 (Supplemental Table 8); two linkage groups were assigned to chromosome 4 and two to chromosome 6 based on common markers with the BYG15-23 \times AC43 family (described below). SNPs showing significantly ($P < 0.001$) distorted segregations in the OH1 \times 5225 haploid family were concentrated on chromosomes 7 and 8 (Fig. 1; Supplemental Table 8). Many of these distorted markers segregated normally in the BYG15-23 \times AC43 family and had essentially the same linkage orders (Fig. 1), indicating that these two genomic regions were likely under selection among haploids from the OH1 \times 5225 family. Two small regions on the ends of chromosomes 1 and 6 also showed segregation distortions in the OH1 \times 5225 family (Fig. 1).

A total of 479 (342 phase-known and 137 phase-unknown) markers segregated in the BYG15-23 \times AC43 family, of which 339 were new SNPs identified in this research and 140 were previously developed RFLPs, SNPs, or SSRs (Martin et al. 2005), yielding ten linkage groups at LOD ≥ 4.0 (Supplemental Table 9). Of the SNPs newly identified in this research, 223 also segregated in the haploid families (Supplemental Table 10). The orders of co-segregating SNPs were similar for the OH1 \times 5225 and BYG15-23 \times AC43 families, although marker orders on chromosomes 2 and 6 were inverted between the two families over relatively short genetic distances (Fig. 1). Common SNPs between these two segregating families allowed for the assignment of linkage groups from OH1 \times 5225 to chromosomes (Fig. 1). Eighteen (4 %) markers in the BYG15-23 \times AC43 family showed significant ($P < 0.001$) segregation distortion and many were located on chromosome 5. Again, many of the SNPs in these distorted regions segregated normally in the OH1 \times 5225 family and their marker orders were essentially the same (Fig. 1).

Discussion

Due to the extremely large nuclear genome of onion, sequencing of random genomic fragments revealed only

4 % of shot-gun reads showing significant similarities to non-organellar proteins (Jakše et al. 2008). Alternatively, transcriptome sequencing has proven to be an efficient approach to sample lower-copy regions of the onion genome (McCallum et al. 2001). Kuhl et al. (2004) completed 20,000 single pass sequencing reactions from the 5' end of cDNAs from a normalized library of onion, and these expressed sequences were a good source of SSRs and SNPs for mapping (Martin et al. 2005). Baldwin et al. (2012b) recently reported on transcriptome sequencing of normalized cDNA libraries from doubled haploid and open-pollinated populations of onion. Their results supported transcriptome sequencing as an efficient approach to reveal DNA polymorphisms (SNPs, indels, and cleaved amplified polymorphisms) in expressed regions of the onion genome. We used the same approach as Baldwin et al. (2012b), completing 454 sequencing from two normalized cDNA libraries from two inbred lines (5225 and OH1) of onion. Like Baldwin et al. (2012b), we chose the 454 platform because of longer read lengths in order to aid assembly of random reads. Over 1 billion bases of expressed sequence from onion were generated from our two libraries. All sequences are freely available from Genbank (Sequence read archives SRX188612 and SRX188621 and Transcriptome Shotgun Assemblies GAAN000000000 and GAAO000000000). Numbers of reads from our libraries were twice the number generated by Baldwin et al. (2012b), assembling into over 48,000 contigs (Table 3).

We identified highly confident SNPs by requiring that multiple reads supported variants in OH1 and 5225. Primers flanking 1,256 putative SNPs were synthesized, of which 930 consistently produced amplicons across the diverse set of onion DNAs listed in Table 2, yielding the largest number of SNPs identified to date for onion. We chose to use the KASPar assay to genotype these SNPs (Supplemental Table 6) because this platform is most commonly used by seed companies. Importantly 930 SNPs were present among the evaluated DNAs (Supplemental Table 7), indicating that they will be useful for diversity studies and fingerprinting of onion germplasms. Significant heterozygosity (9.8 and 19.9 %) was revealed within OH1 and 5225, respectively, as expected because onion inbreds retain heterozygosity due to significant inbreeding depression (Jones and Davis 1944). 5225 was chosen as a putative doubled haploid for sequencing and mapping; however this line was heterozygous at many more SNPs than the ~ 1 % observed in doubled haploids 2107 and H6 (Supplemental Table 7). Heterozygous SNPs in 5225 were concentrated at the ends of linkage groups (Supplemental Table 11), indicating that the cell in the female gametophyte that gave rise to 5225 may have undergone second division restitution (Ramanna and Jacobsen 2003). The approximately 1 % heterozygosity in doubled haploids 2107 and H6 likely

arose from amplifications from duplicated regions of the onion genome (King et al. 1998).

We exploited gynogenic haploids for efficient genetic mapping of SNPs. Gynogenic haploids offer numerous advantages for mapping of genetic markers in onion, including rapid development of segregating families and the advantage that each haploid plant represents a gamete. CsCl-purified DNAs from the haploid family can be stored over the long term for use by other research groups for mapping of additional markers and joining of different genetic maps. However, challenges include the difficulty of extracting haploids from many onion populations and doubling chromosome numbers in order to seed propagate progenies (Jakše et al. 2010). The relatively low heritability of gynogenic haploid production is well documented in onion (Bohanec et al. 2003). Because OH1 was selected for high gynogenic haploid production (Havey and Bohanec 2007), we hoped that F_1 plants from the cross of OH1 by 5225 would efficiently produce gynogenic haploids showing low segregation distortion. However, this was not observed. Distorted ratios ($P < 0.001$) were observed for 35 % of the SNPs segregating in the haploid family, as compared to 11 % in the sexually produced F_2 family from BYG15-23 \times AC43 (Supplemental Tables 8 and 9). Segregation distortion across specific chromosome regions has been commonly observed among haploid progenies (Rivard et al. 1996; Tai et al. 2000).

Flow cytometry identified 11 diploids out of a random sample of 96 gynogenic progenies from OH1 \times 5225; this frequency of spontaneous diploid plants from the female gametophyte is close to the previous report of 10 % by Bohanec (2002). The diploids were homozygous for all segregating SNPs indicating that they likely arose from spontaneous doubling of a cell in the female gametophyte, and not from maternal tissue (all polymorphic SNPs would be heterozygous) or from restitution gametes (a proportion of polymorphic SNPs would remain heterozygous).

A total of 713 newly identified SNPs were placed on the genetic map of onion: 597 in the OH1 \times 5225 family and 339 in the BYG15-23 \times AC43 family. Two hundred and twenty-three SNPs segregated in both families and were used to join the two maps together (Fig. 1) and assign linkage groups to chromosomes (Martin et al. 2005). Numerous aberrantly segregating SNPs ($P < 0.001$) in the OH1 \times 5225 or BYG15-23 \times AC43 families segregated normally in the other family, mapped to the same linkage groups, and were largely syntenic (Fig. 1). This observation indicates that different chromosome regions were under selection during the extraction, growth, or propagation of gynogenic haploids from OH1 \times 5225, or during selfing to produce the BYG15-23 \times AC43 family, resulting in skewed segregation ratios across specific regions.

Significant effort has gone into the development and mapping of molecular markers in onion, including RFLPs (King et al. 1998), randomly amplified DNAs (Bradeen and Havey 1995), AFLPs (Van Heusden et al. 2000), SSRs (Jakše et al. 2005; Martin et al. 2005; McCallum et al. 2008; Baldwin et al. 2012a), and SNPs (Martin et al. 2005; McCallum et al. 2008; Baldwin et al. 2012b). Our research has produced the largest number of robust, commonly occurring SNPs in onion, adding significantly to the 43 and 93 SNPs mapped by Martin et al. (2005) and Baldwin et al. (2012b), respectively. All eight chromosomes of onion are relatively well covered by these newly identified SNPs (Fig. 1). Because these SNPs are in expressed regions of the genome and commonly occur among elite germplasms, they will be useful for the development of high throughput genotyping platforms for gene tagging, marker-aided selection, and fingerprinting of onion.

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